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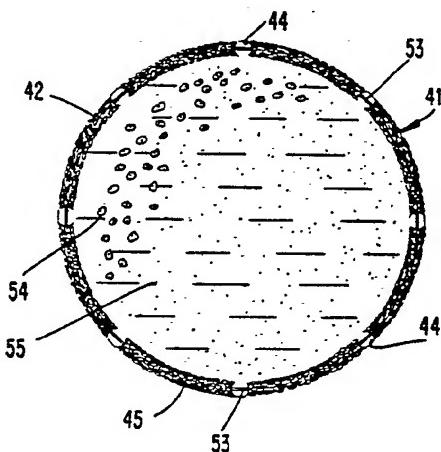
(57) Abstract

A bioreactor comprising a hollow microsphere container (41) and a liquid suspension or solution (55) of biocatalyst (54) encapsulated with the hollow microsphere. The hollow microsphere comprises a spherical porous wall (45) comprised of sintered together inorganic particles (42). The microsphere wall is of substantially uniform thickness and porosity, the wall includes interconnecting voids which extend from the outer surface of the wall to the inner surface of the wall and the wall encircles an intracapsular space in the form of a single hollow central void. The intracapsular space is at least 70% filled with the biocatalyst solution or suspension. The microcapsule wall further comprises a plurality of macropore openings (44) extending through the thickness of the wall and having a diameter larger than the major dimension of the biocatalyst, and a semipermeable membrane (53) having a thickness substantially less than the thickness of the microsphere wall. The membrane extends across the interconnecting voids and the macropores in order to encapsulate the biocatalyst within the intracapsular space, but permitting diffusion of gases, liquids and dissolved or suspended substances having a molecular weight below a predetermined molecular weight cut-off.

HOLLOW POROUS MICROSPHERE BIOREACTORS AND BIOCHEMICAL PROCESSES USING SAME

Related Applications

This application is a continuation-in-part of Application Serial No. 639,126, filed August 9, 1984, titled "Hollow Microspheres Made From Dispersed Particle Compositions and Method and Apparatus for Producing Them," the disclosure of which is incorporated herein in its entirety by reference thereto.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to novel hollow porous microspheres having the pores thereof closed, e.g. coated, impregnated, sealed, filled or covered with a fluid permeable substance, and to the use of these microspheres for providing a non-rupturable support and protective environment for a biocatalytic substance, e.g. microorganisms, cells, cellular products, enzymes, antibodies, and other biochemically active materials, and to the use of the encapsulated biocatalysts, as bioreactors in biochemical processes.

More particularly, the invention relates to bioreactors, i.e. encapsulated biocatalysts, such as, for example, enzymes, antibodies, hormones, interferons, lymphokines, and other non-living biologically active substances, as well as various living substances, including viruses, bacteria, yeasts, single cells, organelles, cellular masses, tissues, and the like, wherein the encapsulating containers are in the form of non-rupturable hollow porous microspheres wherein the pores are coated, impregnated, sealed, filled, covered, or otherwise closed-off with a fluid permeable gel and/or permselective membrane material; and to the biochemical processes using the novel bioreactors.

Discussion of the Prior Art

A bioreactor may be broadly defined as a biocatalyst in a container. The biocatalyst may be any one of a wide variety of chemically active biological substances, such as, for example, enzymes, hormones, antibodies, interferons, lymphokines, and other non-living substances, and living microorganisms, such as bacteria, viruses, yeast, single cells, organelles, cellular masses, tissues, etc.

Traditionally, bioreactors have taken the form of fermentation vats, as in the production of alcohols, sugars and other fermentation products. Recently, as the containers or supports for these biocatalysts porous solid and porous hollow organic and inorganic fibers, and microcapsules have been proposed and have been commercially available. The porous fibers and microcapsules provide the advantage of large surface areas for a small volume and when the biocatalyst is encapsulated in the fiber or microcapsule, a sterile environment. A general discussion relating to the use of bioreactors in biotechnological processes can be found in Science, Vol. 219, February 11, 1983, pp. 728-733, "Bioreactors: Design and Operation," Charles L. Cooney.

As representative of the U.S. Patent art relating to hollow fiber membrane cell culture devices mention can be made of the following: 3,821,087, 3,883,393, 3,997,396, 4,087,327, 4,184,922, 4,189,534, 4,200,689, 4,201,845, 4,266,026, 4,293,654, 4,301,249, 4,440,853 and 4,442,206.

As representative of the U.S. Patent art relating to organic polymeric porous microcapsules for encapsulating various biocatalysts mention can be made of the following: 3,522,346 - Chang; 4,349,530 - Roper; 4,353,888 - Sefton; 4,148,689 - Hino, et al; 4,310,554 Olson, et al; 3,767,790 and 3,860,490 - Guttag; 4,431,428 - Schmer; 4,321,327 - Chen, et al; and the following group of patents, all assigned to Damon Corporation: 4,251,387, 4,255,411, 4,257,884, 4,322,311, 4,324,683, 4,352,883, 4,391,909, 4,407,957 and 4,409,331.

There is also a large body of patent art relating
to the use of solid beads, which may or may not be porous,
on which microorganisms and/or enzymes can be fixed or
immobilized. For example, mention can be made of the
following U.S. Patents: 4,343,901 - DeFilippi; 4,189,534
and 4,293,654 - Levine, et al; 4,153,510 Messing; 3,717,551 -
Bizzini, et al, and others. See also, K. Nilsson, et
al., "Entrapment of animal cells for production of monoclonal
antibodies and other biomolecules," Nature, Vol. 302,
Apr. 14, 1983, pp. 629-630.

U.S. Patent 3,875,008 to Yoshino, et al describes
the encapsulation of enzymes and/or microorganisms in
the lumen of a hollow semi-permeable polymeric filament
which may be opened or closed at its ends.

The hollow fiber permselective membranes have
the disadvantages of requiring headers which are difficult
to manufacture and seal, frequent breakage, and especially
for the organic fibers, inherently slow separation rates
due to the requirement of having to use relatively thick
separation membranes to obtain the necessary strength
required to support the weight of the membranes. Moreover,
the hollow fiber cell culture devices generally function
primarily as supports and merely adhere the cells or
other biocatalysts to the outer surface of the hollow
fiber or in the pores of spongelike hollow-fibers or
in the pores of solid porous microspheres or microbeads,
and therefor do not provide a sufficiently sterile environment.

The hollow semipermeable organic microcapsules
and gelatinous microbeads and the hollow semipermeable
fibers which encapsulate cells, bacteria or other biocatalysts
can provide an adequate sterile environment but suffer
from the defect of inherently slow permeation rates due
to the wall thickness of the membrane. However, even
where the biocatalyst is encapsulated within the lumen
of a hollow fiber, as in the above mentioned Yoshino,
et al patent, there is still the risk of fiber breakage
which can contaminate and render useless the entire or
substantial portion of a production run. In addition,
to date no practical economical method has been developed

to fabricate the hollow microcapsule containers with
uniform size or wall thickness and therefore control
of the biotech processes using bioreactors based on these
hollow permselective microcapsules is extremely difficult.
Still further, these organic microcapsules and gels are
generally fragile and cannot be stacked to any significant
height, or used in, for example, a fluidized bed process,
since the weight of the column or particle-to-particle
or particleto-wall contact can rupture, distort, or otherwise
damage and ruin the microcapsule containers and gel beads.

Objects of the Invention

Accordingly, it is an object of the invention
to provide bioreactors from inexpensive, easily manufactured,
reusable or discardable, uniformly sized, high strength,
porous hollow microspheres.

It is another object of this invention to provide
structural support to gel immobilized biocatalyst without
significantly diminishing permeation rates of nutrients,
gases, reaction substrates or other desired substances
into and out of the gel by encapsulating the immobilized
biocatalyst in high strength hollow uniformly sized porous
microspheres.

Another object of the invention is to provide
biocatalysts encapsulated within rigid thin walled hollow,
porous microspheres, the walls of which are uniform in
diameter, thickness, porosity, and interconnecting void
distribution, and which walls contain at their outer
surface, inner surface, and/or intermediate location,
a permselective membrane having a predetermined molecular
weight cut-off wherein the biocatalysts are protected
from contamination or inactivation by high molecular
weight substances by the permselective membrane which
effectively separates the hollow interior of the microsphere
from its external surroundings with respect to high molecular
weight substances.

A more specific object of the invention is to
provide novel bioreactors for biotech processes wherein

the containers of the bioreactors are hollow porous microspheres
as support for permselective membranes wherein substantially
the entire structural strength of the bioreactor is provided
by the walls of the microspheres such that the permselective
membranes can be substantially thinner than in conventional
permselective membrane bioreactors, that is, in which
the structural support function is separated from the
permselective membrane function.

It is also an object of this invention to provide
a process for encapsulating biocatalyst substances within
non-rupturable hollow porous thin walled microspheres.

A still further object of this invention is to
provide biotech processes for manufacture, purification,
separation, and identification of various chemical substances
which use the novel bioreactors.

Yet another object of the invention is to provide
more economical, easier to control biotech process using
uniformly sized hollow porous non-rupturable microsphere
supported permselective membrane encapsulated biocatalyst
as bioreactor.

Still yet another object of the invention is
to provide microsphere encapsulated biocatalyst bioreactors
which can be used in fixed bed, fluidized bed, and other
batch, continuous or semi-continuous biotech processes.

These and other objects of the invention will
become apparent as the description proceeds.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides
novel bioreactors which include, as the containers for
containing and protecting the biocatalyst, hollow porous
microspheres of substantially uniform diameter and substantially
uniform wall thickness and porosity, and high mechanical
strength, the porous wall or shell of the hollow microsphere
including entrance means through which biocatalyst is
introduced into the hollow interior or core of the microsphere,
biocatalyst in the hollow interior of the microsphere,
and fluid permeable means for immobilizing the biocatalyst

within the hollow interior.

In one embodiment of the invention, the fluid
permeable immobilizing means can be a reversible or irreversible
gel. Where the biocatalyst is a living microorganism,
the immobilizing means preferably includes a continuous,
semicontinuous or discontinuous permselective membrane
sealing the pores and the entrance means in the wall
of the microsphere, such that the biocatalyst contained
within the microsphere is prevented from escaping from
the microsphere through the pores and entrance means,
while only specific liquids, gases and/or organic molecules
of predetermined molecular size, which is smaller than
the size of the biocatalyst, can enter or leave the microsphere
through the permselective membrane. The permselective
membrane sealed microsphere containers can be used with
any biocatalyst, such as enzymes, however, they are especially
preferred when the biocatalysts are living viable microorganisms,
including single cells, bacteria, viruses, fungi, molds,
and yeast, both naturally occurring and modified, fused
or genetically engineered.

In another aspect, the invention provides a process
for producing the bioreactors from the hollow porous
microspheres. According to this process, hollow porous
microspheres each having entrance means in its porous
wall or shell which is large enough for the biocatalyst
to pass through into the hollow interior, are suspended
in an inert or nutrient containing liquid medium, in
which liquid medium the biocatalyst and a gel-forming
reactant is dissolved or suspended, and sufficient pressure
is applied to the resulting suspension to cause the dissolved
or suspended biocatalyst and gel-forming reactant and
the liquid medium to pass through the entrance means
of the microsphere into the hollow interior of the microsphere.

In one embodiment of the process, the biocatalyst -
gel forming reactant - filled microspheres are recovered
from the suspending medium and placed in a second medium
which causes the gel forming reactant to gellify, at

least in the pores and entrance means whereby the resulting
gel, as supported by the microsphere wall, encapsulates
the biocatalyst within the intracapsular volume of the
microsphere to thereby provide the fluid permeable immobilizing
means.

In the embodiment of the invention wherein the
fluid permeable immobilizing means is comprised of a
semipermeable membrane material the gel immobilized biocatalyst
filled microspheres are further treated with a membrane
forming material whereby the previously formed gel in
the entrance means and pores functions as a substrate
on which the membrane is deposited by mechanical, chemical
or mixed mechanical-chemical means. According to this
embodiment, after recovery of the gel immobilized biocatalyst-filled
microspheres, the biocatalyst-filled microspheres are
contacted with a solution or suspension of the material
forming the permselective membrane under conditions which
will cause the membrane material to deposit on and/or
react with the gel to fill, coat, seal, impregnate or
otherwise close-off the entrance means and substantially
all of the pores of the microspheres, thereby preventing
the biocatalyst from escaping from the interior of the
hollow microspheres while permitting diffusion of substances,
such as nutrients, reactants, by-products, and the like,
of predetermined molecular size through the permselective
membrane into or out from the interior of the biocatalyst-filled
microspheres. The gel may optionally be reliquefied to
facilitate the activity of the biocatalyst. Where necessary,
as might be the case, for example, with polymeric permselective
membranes deposited from a solution of the polymer, the
coated microspheres are recovered and the solvent is
removed under sufficiently mild conditions to avoid inactivating
the biocatalyst.

According to still another aspect of this invention
an improved method for the biochemical production, purification
or separation of useful substances or the identification
and/or quantification of one or more substances in a

mixture is provided in which the improvement involves
the use of the novel bioreactors as described above.
The bioreactors are maintained in an environment conducive
to the production, purification, separation, identification
and/or quantification of the desired substance but without
degradation of the biocatalyst. In the case of chemically
active biological materials, such as enzymes, hormones,
antibodies and the like, the biocatalyst-filled micro-
spheres are maintained in contact with a medium containing
at least one low molecular weight material which is reactive
with the biocatalyst and which reactive material(s) can
diffuse through the gel or permselective membrane fluid
permeable immobilizing means to react with the encapsulated
biocatalyst. Depending on the molecular size of the
desired end product, the end product can be recovered
from the medium external to the microspheres or the microspheres
with the encapsulated high molecular weight end product
can be mechanically or chemically opened or the gel or
permselective membrane can be dissolved or disrupted
outside the reaction system to recover, detect and/or
quantify the end product.

In the preferred embodiment of the invention
in which the biocatalysts are in the form of living microorganisms,
such as single cells, cell tissues, bacteria, viruses,
yeast cells, and the like, and wherein a semipermeable
membrane is used as the fluid permeable immobilizing
means, the biocatalyst-filled microspheres are maintained
in contact with a nutrient medium for the particular
organism under culture conditions in which the organisms
will generate the desired useful end product, usually
an amino acid, nucleic acid, nucleotide, phosphatide,
sugar, protein, glycoprotein, etc. Any nutrients necessary
for maintenance and/or growth of the cells or other micro-
organisms but which have a molecular weight too large
to pass through the permselective membrane will be included
within the microsphere prior to formation of the permselective
membrane. Where the end product is excreted from the
microorganism and is of sufficiently small molecular

size so that it can diffuse through the permselective
membrane, the end product can be recovered from the external
liquid nutrient medium. Where the end product is merely
accumulated in the organism, as is the case, for example,
with certain bacterial and yeast produced amino acids,
proteins, etc., or where the molecular size of the end
product is too large to diffuse out of the microspheres,
the microspheres are withdawn from the nutrient medium
and the end product is recovered by rupturing the walls
of the microspheres or by removing, e.g. dissolving,
the permselective membrane. In the latter case, the
bioreactors can be recycled after replacing the permselective
membrane and, if necessary, introducing fresh biocatalyst.

The invention will now be described in greater
detail by specific embodiments and examples and with
the aid of the accompanying drawings in which:

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is an enlarged cross-sectional view of
a microsphere container according to the invention;

Fig. 2 is an enlarged cross-sectional view of
a modified embodiment of the microsphere container including
a multiplicity of macropores extending through the microsphere
wall;

Fig. 3 is similar to Fig. 1 but showing the intracapsular
space of the microsphere filled with a solution or suspension
of biocatalyst, such as an enzyme;

Fig. 4 is similar to Fig. 2 but showing the intra-
capsular space of the microsphere filled with a suspension
of cellular biocatalyst and further showing a permselective
membrane;

Fig. 5 is an exploded schematic view of a segment
of the microsphere wall of Fig. 1;

Fig. 6 is an exploded view of a section of the
microsphere wall of Fig. 4 including the permselective
membrane; and

Fig. 7 is a microphotograph (900x) of a section
of a microsphere wall showing a macropore therein.

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DETAILED DESCRIPTION OF THE INVENTION
AND PREFERRED EMBODIMENTS

Hollow Porous Inorganic Microspheres

The hollow porous inorganic microspheres provide the uniformly sized containers for the biocatalyst and the structural support for the permselective membrane. Suitable methods for manufacturing these microspheres and their physical properties and dimensions are disclosed in and are the subject matter of applicant's copending application serial no. 639,126 "Hollow Porous Microspheres And Method And Apparatus For Producing Them" filed on August 9, 1984. The entire disclosure of the copending application is incorporated herein in its entirety by reference thereto.

Quite briefly, the hollow porous microspheres are made from aqueous or non-aqueous suspensions or dispersions of finely divided inorganic or organic solid particles, particularly ceramic, glass, metal and metal glass particles, having particle diameters in the range of from about 0.01 to 10 microns (μ), a binder material, a film stabilizing agent, a dispersing agent for the solid particles, and a continuous aqueous or non-aqueous liquid phase. The suspension or dispersion is blown into microspheres using a coaxial blowing nozzle, the microspheres are heated to evaporate the solvent and further heated or cooled to harden the microspheres. The hardened microspheres are then subjected to elevated temperatures to decompose and remove the binder and any residual solvent or low boiling or melting materials. The resulting porous hollow microspheres are then fired at further elevated temperatures to cause the particles to sinter and/or fuse at the points of contact of the particles with each other such that the particles coalesce to form a strong rigid network (lattice structure) of the sintered-together particles.

The hollow porous microsphere containers of this invention, in addition to their uniformity in structure,

have the highly advantageous characteristic of high mechanical
strength due to the sintering together of the solid dispersed
wall-forming particles. Due to this high mechanical
strength, the microsphere containers are non-rupturable
under the conditions of actual use in biotech processes.
Thus, as used herein, and in the appended claims, "non-rupturable"
means the ability to withstand the forces exerted by
contact with other microspheres or the walls and surfaces
of the biotech apparatus, as well as hydrostatic forces
and pressures encountered in biotech processes, including
fluidized bed, stacked bed, plug flow, and other types
of biotech processes, without any significant deformation
of shape and without breakage and, further, without imparting
stress forces on the permselective membrane or other
fluid permeable immobilizing means. Preferably the walls
of the hollow porous microsphere containers are rigid
and are capable of withstanding two point contact pressure
of at least 30 psi (2.1 kg/cm^2), preferably from about
50 psi (35 kg/cm^2) to about 300 psi (21.1 kg/cm^2) and
hydrostatic pressure of at least 150 psi (10.5 kg/cm^2),
preferably from about 150 psi (10.5 kg/cm^2) to about
1500 psi (105.7 kg/cm^2). As used herein "two point contact
pressure" is measured with respect to a one-inch square
tightly packed monolayer of the microspheres resting
on a hard flat surface with a flat mass placed thereon.
The weight of the mass causing breakage of one or more
microspheres divided by one square inch is the "two point
contact pressure."

          As can be seen from Figures 1 and 5, the sintered-
together particles 42 forming the solid porous wall 45
of the microsphere 41 define, within the wall, interconnecting
voids or channels 46. For simplicity of illustration,
the particle-to-particle contact of the sintered together
particles is not shown. These interconnecting voids
are continuous and extend, often in tortuous or curved,
but smooth paths, from the surface pore or opening 47
at the outer wall surface to the surface pore or opening

48 at the inner wall surface, thereby providing generally
tortuous, but smooth, i.e. without excessively sharp
bends or curves, paths or passageways for transporting
gases, liquids and low molecular weight solutes and very
finely divided, e.g. submicron, dispersed solid materials,
from between the exterior (referred to as "extracapsular"
space) 49 to the single hollow central void, i.e. interior
(referred to as "intracapsular" space) 50 of the hollow
microsphere. The interconnecting voids are substantially
uniformly distributed in the walls of the hollow microspheres
(referred to as "uniform void distribution") while the
microspheres, because of the unique production process,
also have a substantially uniform porosity (also referred
to as "void content") and diameter and wall thickness
from microsphere to microsphere.

Generally, the microspheres will have an outer
diameter of about 200 to 10,000 microns, preferably about
1200 to 6000 microns, and more preferably about 2000
to 4000 microns and a wall thickness of about 1 to 1000
microns, preferably 10 to 200 microns and more preferably
20 to 100 microns. The ratio of the wall thickness to
outer diameter can be in the range of from about 1:4
to 1:500, preferably 1:10 to 1:300, and more preferably
1:50 to 1:200. The porosity of the walls is generally
from about 5 to 45%, preferably 15 to 35%, and more preferably
25 to 30% by volume of the microsphere wall. The interconnecting
voids or channels will, depending on the size of the
dispersed particles and the particle size distribution
of the dispersed particles and the porosity of the microsphere
walls, range from about 0.05 to 8 microns, generally
from about 0.1 to 5 microns, and more generally from
about 0.1 to 3 microns.

For most non-living biocatalysts, e.g. enzymes,
antibodies, lymphokines, etc., having molecular weights
of less than about 100,000 to 200,000 daltons, the pore
sizes or at least a substantial portion of the pores,
will be sufficiently large to permit free passageway
of these biocatalysts from the extracapsular space into

the intracapsular space (the hollow core) of each microsphere.

Thus, as illustrated in Fig. 3, enzymes 52 dispersed
in liquid medium 51 fill the intracapsular space 50 through
the channels 46 between the sintered together solid particles
42.

However, the pore size may be smaller than or
only slightly larger (e.g. up to about 100% larger) than
the maximum dimension of the biocatalyst - this will
generally be the case for cells, tissues, bacteria, yeast
cells, and other microorganisms. In such a case, the
microspheres illustrated in Fig. 2 can be used. As seen
in Fig. 2, at least one, and preferably about 5 or more
entrance means 44, e.g. openings or passageways, of larger
dimensions than the largest pore diameter, are provided
to ensure that the cellular biocatalyst 54 suspended
in broth 55 will be able to pass through the entrance
means 44 which extend through the wall 45 of the microsphere
41 into the intracapsular space 50 of the microsphere
prior to the depositing or formation of the gel or permselective
membrane immobilizing means 44. The relatively large
entrance means in the walls of the microspheres, e.g.
which are at least twice as large as the maximum pore
size, are referred to herein as "bioports" or "macropores."
However, it is understood that the term "entrance means"
as used herein, and in the appended claims, can also
include the pores and interconnecting voids in those
cases where the biocatalyst dimensions are substantially
smaller, e.g. 50% or less, than the dimensions of the
pores and interconnecting voids and can, therefore, freely
pass from the extracapsular space to the intracapsular
space. Preferably, however, even with the smaller dimensioned
biocatalysts, it is preferred to include one or more
macropores 44 in the microsphere walls to facilitate
and expedite the process of filling the microspheres
with biocatalyst and subsequently removing the biocatalyst.

As described in the copending application serial
no. 639,126, the macropores can be obtained by incorporating

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in the solid particle suspension or dispersion, prior
to the blowing step, a small percentage of decomposable
particles (macroparticles) having a diameter greater
than the maximum dimension of the biocatalyst, for example,
about 1 to 1000 µ, preferably 5 to 200 µ, more preferably
about 10 to 200 µ, especially preferably about 20 to
100 µ. These decomposable macroparticles are entrapped
along with the smaller dispersed solid particles in the
wall or shell of the microsphere. However, the decomposable
macroparticles are decomposed at the step of decomposing
the organic binder or at the subsequent step of sintering
the dispersed particles depending on the decomposition
temperature of the decomposable macroparticles leaving
behind large openings (macropores), such as shown in
figure 2.

Fig. 7 is a microphotograph (900x magnification)
showing a section of a microsphere wall (40 micron thick)
after decomposition of acrylic macroparticles of about
50 µ diameter. The sintered solid particles forming
the walls are alumina (Al_2O_3) particles having a particle
size in the range of about 1 to 3 microns. The microsphere
is about 4000 microns in diameter.

Examples of materials from which the decomposable
macroparticles can be formed include, for example, carbon,
naphthalene, anthracene, camphor, polyformaldehyde resins,
polyethylene, polypropylene, nylon, and, more generally,
any of the thermoplastic or thermosetting organic resins
or polymers which can be used as the binder, provided
however that the decomposable macroparticles are not
soluble in and do not swell or substantially swell in
the solvent for the binder. In addition, metal and glass
beads or pellets having a melting temperature below the
sintering temperature, preferably at least 100°C below
the sintering temperature can also be used.

As can be seen from the microphotograph of Fig. 7, the perimeter of the macropore is generally free of sharp or jagged protrusions which could result from extension into the macropore of portions of one or more of the finely divided solid wall-forming particles since any such sharp or jagged protruding solid particles will tend to be smoothened by the subsequent sintering step. However, it is possible to even further smoothen the surface of the macropore by using as the macroparticles a material, such as glass and metals, which at least partially soften and melt, rather than decompose, at the operating temperature. In such case, when the microsphere is heated, at least a portion of the macroparticle will diffuse and penetrate into and between the dispersed solid particles surrounding and coating the macroparticle thereby assuring leaving behind a smooth macropore surface (periphery). Furthermore, it is possible to select mutually reactive materials for the finely divided solid particles and the macroparticles, for example, alumina solid particles and glass macroparticles, which will react to form alumina silicate, at or below the sintering temperature, thereby further strengthening, as well as smoothening, the macropore surface.

The amount of the decomposable particles incorporated in the suspension or dispersion is not particularly critical insofar as the amount is sufficiently high so that all of the formed porous hollow microspheres contain at least one, preferably at least 5, and especially preferably at least about 10 to 20 decomposable particles in their walls. On the other hand, the amount of decomposable particles should not be so high that the blowing operation is impeded or that the mechanical strength of the microsphere wall is weakened. Generally, the amount of the decomposable particles will range from about 1 to 10%, preferably from about 2 to 6%, especially preferably from about 2 to 4%, by volume of the total volume of dispersed solid particles, to satisfy the above requirements.

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Generally, the material of the dispersed solid
particles forming the walls of the microspheres is not
particularly critical, so long as it is compatible with
and non-toxic to the biocatalyst and inert to the biotech
process, and any of the ceramic particles, glass particles,
metal particles, metal glass particles, and plastic particles
disclosed in the aforementioned patent application serial
no. 639,126 can be used.

On the other hand, it is often preferred or necessary
in certain biotech processes for the biocatalyst to adhere
to a substrate (in the case of the present invention,
the substrate being the inner wall surface of the hollow
microsphere, as well as the portions of the semipermeable
membrane exposed to the intracapsular space). For example,
as is well known in the art, mammalian cells are generally
anchorage dependent for growth (cell division). Also,
it is often preferred to immobilize or fix enzymes to
substrates in biotech processes. In such cases, therefore,
the material of the dispersed particles will be selected
based on its ability to provide a surface to which the
biocatalyst can adhere by physical and/or chemical bonding.
Most materials will naturally meet this requirement,
although to differing degrees. Furthermore, it is also
known in the art to provide chemical treatment to substrates
to increase their ability to bond to specific biocatalysts.

It is preferred that, for reasons discussed herein,
the wall forming material, i.e. solid particles, should
be inorganic rather than organic.

An inorganic material has a variety of marked
advantages. The first is that microbes do not readily
attack inorganic materials since their nutrient requirements
are primarily focused to carbon and nitrogen containing
materials. Organic substances, such as carbohydrates,
proteins, etc., are readily attacked not only by the
microbes but also by the extracellular enzymes that are
elaborated by the organism. As the organic particles
are destroyed, the accumulation of microbes is reduced.
In addition to durability, the inorganic microspheres

have the advantage of dimensional stability and mechanical strength when contrasted to organic microspheres. By retaining the pore morphology under a variety of pressure and flow conditions, the microsphere is protected from deformation. Again, this is an advantage in terms of product formation biomass accumulation and/or process control.

An additional advantage of an inorganic microsphere is its relatively high density. Most organic materials have densities in the neighborhood of 1.0 or less while most of the inorganic materials have densities greater than 2.0. Under these circumstances, inorganic material microspheres having desired bulk densities approximating that of the aqueous nutrient culture broth or other liquid system can be more easily obtained.

On the other hand, as compared to the known solid porous microbead carriers used as biocatalyst supports, the hollow porous microspheres according to this invention have significantly lower densities. These low densities (or mass) provide the following highly important advantages: less mechanical energy is required to mix or stir suspensions of the microsphere bioreactors in biotech processes thereby providing lower overall costs for carrying out the process. The lower bioreactor mass reduces the impact forces or collision pressures of particle-to-particle, i.e. microsphere-to-microsphere, collisions, thereby reducing the likelihood of damage to the microsphere walls and more importantly, due to the inherent strength of the microsphere walls, reducing the likelihood of damage or shearing stresses to the permselective membrane or other fluid permeable immobilizing means. As is generally known in the art, stresses applied to permselective membranes can alter the molecular weight cut-off point of the membrane and thereby make control of the biotech process more difficult.

Mixtures of two or more materials forming the dispersed particles can also be used, provided, however, that the different composition particles can be sintered

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to each other without melting or fusing the particles
to such an extent as to substantially decrease the porosity
of the microsphere to below about 5%, preferably 15%.

Biocatalysts

Any chemically active biological substance or
living cells or microorganism can be used as the biocatalyst
which is to be encapsulated within and protected by the
hollow porous microspheres.

As examples of chemically active biological substances
mention can be made, for example, of enzymes, such as
carbonic anhydrase, urease, asparginase, lactate dehydrogenase
(LDH), glutamate oxaloacetate transaminase (GOT), aminoacylase,
aspartase, esterase, fumarase, glucose isomerase, lactase,
penicillin acylase, racemase, steroid dehydrogenase,
etc., hormones, antibodies, hemoglobin, interferons,
lymphokines, such as interleukin-1 and interleukin-2,
macrophage activating factor (MAF), colony stimulating
factor (CSF), etc., and other chemically active materials
including, for example, ion exchange resins and activated
charcoal. In place of cell-free enzymes, ruptured cells
or whole cells which produce the desired enzymes can
also be used. Mixtures of biocatalysts can also be used.

In addition to the aforementioned examples of
non-living biocatalyst materials, there are many biotech
processes which utilize living substances. As examples
of living biocatalysts mention can be made of individual
cells, cellular masses, tissues and organelles, and microbes
or microorganisms including yeast, bacteria, viruses,
fungi, mold and algae. Bacteria and yeast are the preferred
microbes.

The cellular biocatalysts include, for example,
animal cells, especially mammalian cells, including bovine,
porcine, ovine, monkey and human cells, mouse, and hamster
cells; plant cells; and cell masses, fused cells, e.g.
hybridoma cells, and cell tissues from any of these types
of cells. Both normal cells and tumor cells as well
as genetically engineered cells and cells modified by

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conjugation, hybrid DNA, or fusion can be used. Cell
types which are adapted to grow in suspension culture,
as well as anchorage-dependent cells can be encapsulated
in this invention. Specific examples include mammalian
islets of Langerhans, fibroblasts, leukocytes, lymphoblastoids,
pancreatic beta cells, alpha cells, delta cells, various
ratios thereof, bovine and human anterior pituitary cells,
chicken embryo, epithelium, rat liver, and established
cell lines, such as Hela human cervix (carcinoma) cells,
rhesus monkey kidney cells (LLC-MK$_2$), Syrian baby hamster
kidney cells (BHK-21), etc. Of course, any other cells,
tissues, cell lines, etc., capable of being maintained
and/or grown in a suitable culture medium can be encapsulated
in the hollow microspheres according to this invention.

Any of the viruses which can be cultivated by
cell culture techniques in encapsulated form, such as
Rauscher murine leukemia virus, hepatitis A virus, etc.,
can be used in this invention. To cultivate the viruses
they are first inoculated in individual cells according
to known principles and the inoculated cells are then
encapsulated in the hollow microspheres.

Similarly, any type of bacteria or yeast microbes
which can be cultivated in a suitable culture medium
can be used. As non-limiting examples of suitable microbes
mention can be made of, for example, cyan-utilizing microorganism,
pencilin acylase producing microorganism, macrolide type
antibiotic producing microorganism and the like.

As specific non-limiting examples of microorganisms,
there can be mentioned <u>Streptococcus lactis</u>, <u>Saccharoinyces
cerevisiae</u>, <u>Lactobaccillus delbrueckii</u>, <u>Aspergillus niger</u>,
<u>Acetobacterrances</u>, <u>Bacterium curvum</u>, <u>Lactobacillus bulgaricus</u>,
<u>Lactobacillus casei</u>, <u>Lactobacillus leichmannii</u>, <u>Citromyces
pfefferianus</u>, <u>Penicillin arenarium</u>, <u>Rhizopus nigricans</u>,
<u>Penicilin notatum</u>, <u>Streptomyces griseus</u>, <u>Streptomyces
citrovorus</u>, <u>Penicillin roqueforti</u>, <u>Lactobacillus cucumerus</u>,
<u>Lactobacillus bifidus</u>, <u>Lectobacillus acidophilus</u>, <u>Escherichia
coli</u>, <u>Saccharomyces cerevisiae</u>, <u>Saccharomyces anamensis</u>,
<u>Aspergillus oryzae</u>, <u>Saccharomyces ellipsoideu</u>, <u>Saccharomyces</u>

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pastorianus, Clostridium butyricum, Streptococcus lactis,
Propionibacterium freudenreichii, Streptomyces rimosus
forma paromycinus, Actinomyces griseus, Streptomyces
kanamyceticus, Streptomyces humidus, Seratia marcescens,
Bacillus subtilis, and Rhodospirillum rubrum.

"Encapsulation" of Biocatalyst

        The manner by which the biocatalyst is introduced
into the hollow porous microsphere container is not particularly
critical so long as the activity of the biocatalyst is
not substantially diminished or destroyed in the process.
Accordingly, by whatever technique is used, it will be
necessary to maintain an environment which is conducive
to the maintenance of the biological and/or chemical
activity of the biocatalyst, for example, temperature,
pH, and the like, as well, of course, as a suitable nutrient
broth or culture liquid in the case of living cells and
other microorganisms. The appropriate environmental
conditions for any particular biocatalyst will be well
known or readily ascertainable by the practitioner. Usually,
because of the high temperatures to which the microspheres
are subjected during sintering, e.g. about 500°C or more,
it may not necessary to sterilize the microspheres prior
to encapsulating the biocatalyst. Of course, a sterile
environment including all process equipment, should be
maintained until at least after formation of the gel
or permselective membrane.
        Generally, it should be sufficient to merely
suspend the microspheres in a liquid carrier medium in
which the biocatalyst is dissolved, suspended or dispersed,
and to allow the biocatalyst and liquid and any nutrients,
or other substances added to the liquid carrier, to flow,
or to diffuse by capillary action, through the entrance
means or both the entrance means and pores in the walls
of the porous hollow microspheres, depending on the size
of the biocatalyst, into the intracapsular space. The
suspension can be gently stirred by mechanical mixing,

aerobic mixing or other gaseous mixing, to ensure homogeneity
of the system and uniformity of the amount of biocatalyst
in contact with individual microspheres. It is also
preferred that the liquid carrier be capable of wetting
the material forming the walls of the microsphere containers
to assist in the filling process. Wetting agents which
are inert to the biocatalyst can be added to the liquid
carrier medium for this purpose.

The concentration of the biocatalyst in the liquid
carrier and the amount of biocatalyst relative to the
total volume or number of the microspheres will be selected
depending on the nature and type of biocatalyst and the
biotech process and on the internal volume of the microspheres
and can be readily determined by the skilled practitioner.

While the driving force of capillary action can
be sufficient to fill the hollow microspheres with the
suspended, dispersed or dissolved biocatalyst, this technique
requires especially long times, on the order of about
several hours, to fill all of the microspheres, and may
not be practical where the solid particles forming the
microsphere walls and defining the interconnecting voids
or pores and the entrance means are not sufficiently
wetted by the liquid phase of the biocatalyst solution,
suspension or dispersion, since the driving force of
the capillary action may not be sufficient to overcome
the atmospheric pressure within the intracapsular space.

Therefore, according to a preferred embodiment
of the invention, the driving force for filling the hollow
microspheres with the biocatalyst and liquid medium is
increased by applying pressure to the system. The applied
pressure can be fluid pressure, e.g. hydrostatic pressure,
isostatic pressure, pneumatic pressure or dynamic pressure,
e.g. centrifugal force. Preferably, the suspension of
the microspheres in the liquid carrier-biocatalyst system
is subjected to both fluid pressure and centrifugal force
in a centrifuge apparatus.

Generally, the amount of the applied pressure
will not be particularly critical insofar as the pressure

is not so great as to rupture the walls of the microspheres
or harm the biocatalyst and which will maintain laminar
flow of the biocatalyst-liquid medium system. Pressures
in the range of from about 3 psi to about 30 psi, preferably
from about 5 psi to about 25 psi are satisfactory. Preferably,
the pressure is increased gradually since abrupt pressure
increases are more likely to be destructive to the microspheres
and/or biocatalysts. It is usually sufficient to raise
the pressure at a rate of from about 0.5 to about 3 psi/minute,
preferably from about 1 to about 2 psi/minute. The pressure
should be maintained until at least substantially all
of the microspheres are filled with the biocatalyst and
liquid medium. Generally, the time required to fill
the microspheres will be inversely proportional to the
applied pressure and directly proportional to the size
and number of entrance means and pores in the walls of
the microspheres which connect the extracapsular space
to the intracapsular space. Times on the order of from
about 30 seconds to about 60 minutes, generally from
about 1 minute to about 40 minutes are satisfactory.

From an economical point of view, since no special
apparatus is required one preferred filling method is
to simply load a pressure vessel with the microspheres
and thereafter to fill the closed pressure vessel with
the biocatalyst-containing liquid medium under positive
pressure using a suitable pressure pump.

Another relatively simple technique for filling
the hollow microspheres is to "pull" the biocatalyst-liquid
medium system into the microspheres through the entrance
means and/or pores by forming a thin layer, preferably
a single layer, of the microspheres on a microporous
sheet or belt and applying a vacuum to the reverse side
of the sheet or belt whereby the biocatalyst-liquid medium
system will be sucked into the intracapsular spaces of
the microspheres. If desired, a positive pressure can
simultaneously be applied to the biocatalyst-liquid medium
system.

When centrifugal force is used to assist in the filling of the microspheres, a suspension or dispersion of the microspheres in the biocatalyst-containing liquid medium is introduced into a centrifuge tube and rotated at a speed, which depending on the length of the arm carrying the centrifuge tube, will be sufficient to maintain the pressures discussed above. It is also generally preferred to partially fill the microspheres under fluid pressure prior to or during the application of centrifugal force to increase the density of the microspheres relative to the liquid medium.

The amount of the liquid medium to fill the microspheres can be easily calculated based on the volume of the hollow core of a microsphere multiplied by the total number of microspheres. Therefore, the total amount of liquid medium for the filling operation will generally be the volume to fill the microspheres, plus the volume to fill the vessel in which the microspheres are contained less the volume occupied by the microspheres (based on the outside diameter of a microsphere). In other words, assuming the external housing, e.g. pressure vessel, centrifuge tube or other container for the empty microspheres, is filled to capacity with the microspheres (generally about 55-70% of the total volume depending on packing density for a perfect sphere) then the liquid medium can occupy the void volume between individual microspheres plus the additional volume of the hollow cores of the microspheres (neglecting the volume occupied by the interconnecting voids in the walls of the microspheres). Therefore, by metering the amount of the liquid medium supplied to the external housing it can readily be determined when the microspheres are filled with the liquid medium. However, it is generally sufficient to use excess liquid medium and to carry out the filling operation for a period of about 20 to 60 minutes, preferably 30 to 40 minutes to assure adequate filling of the microspheres with the dissolved or suspended biocatalyst and liquid medium.

Furthermore, it is not essential to completely fill the microspheres with the biocatalyst and liquid

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medium and satisfactory results can be obtained when
at least 70%, preferably at least 85%, of the total available
volume of the microsphere is filled, the remaining at
most 30%, preferably at most 15% of the total volume
being filled with air, oxygen, or other gas which is
not detrimental to, or is needed for, the biochemical
process, such as, for·example, oxygen-containing gas
for culturing of mammalian cells and aerobic bacteria.

Biocatalyst Immobilization

After the microspheres are filled with the biocatalyst-
containing liquid medium it is necessary to seal the
entrance means and pores in the walls of the microspheres
in order to immobilize the biocatalyst within the microspheres.

It is a feature of the present invention that
since the microsphere wall, per se, provides all of the
required rigidity and ·structural support for the bioreactor,
it is not necessary for the immobilizing means to contribute
to the mechanical stability and structural integrity
of the bioreactor. Accordingly, it is sufficient to
use any immobilizing means which will effectively retain
the biocatalyst in the intracapsular space while permiting
influx and outflow of gases, nutrients, reactants, by-products,
etc., which are required for the biotech process and/or
for the preservation, maintenance and/or growth of the
biocatalyst.

Thus, in the case, for example, of enzymes, the
immobilization may be effected simply by chemical bonding,
for example, by aldehyde fixation, or any of the many
other techniques known in the art.

A preferred means for immobilizing the biocatalyst
within the microspheres is by encapsulation of the biocatalyst
in a fluid permeable gel formed _in situ_ in the microspheres.
According to this embodiment of the invention, a gel-forming
material or precursor is included in the liquid medium
carrier for the biocatalyst and, after the microcapsules
are filled as described above, the gel-forming material

or precursor is activated to cause gellation. For example,
a water-soluble gum can be used as the gel-forming material.
After the microcapsules are filled, they can be separated
from the liquid medium and transferred to a solution
at gel-forming conditions, for example, temperature,
pH, or ionic conditions. The techniques for formation
of permanent and reversible fluid permeable gels is well
known in the art and any of the conventional techniques
can be used.

In an especially preferred embodiment of the
invention, the biocatalyst is immobilized within the
intracapsular space of the microspheres with a permselective
membrane material which will retain the biocatalyst within
the microsphere but which will allow gases, nutrients,
reactants, wastes, and by-products to pass through or
across the permselective membrane while preventing microorganisms,
such as viruses and bacteria, which are, or may be, harmful
to the biocatalyst, especially living cells or other
microorganisms, or to the biological process, from entering
the microsphere.

According to the preferred embodiment of the
invention, the permselective membrane can be formed in
situ within the entrance means and the pores of the microspheres,
using well-known interfacial polymerization techniques,
such as those disclosed, for example in U.S. Patents
4,324,683, 4,322,311 and 4,251,387 to Lim and Moss, U.S.
Patents 4,352,883 or 4,391,909 to Lim, U.S. Patents 4,407,957
and 4,409,331 also both to Lim, or U.S. Patent 4,353,888
to Sefton, the disclosures of which are incorporated
herein by reference, and many other.

Basically, the above-mentioned interfacial polymerization
techniques can be characterized as forming an aqueous
dispersion or emulsion of fine size droplets - in which
droplets the biocatalyst is itself dissolved or suspended
- in a continuous phase of a hydrophobic liquid medium,
at least one of the aqueous phase and continuous phase
including therein permselective membrane material or
permselective membrane precursor material whereby the

permselective membrane forms at the interface of the
dispersed aqueous phase and the continuous phase whereby
the biocatalyst is encapsulated within a barrier formed
by the permselective membrane.

In applying any of these interfacial polymerization
techniques to the microsphere encapsulated biocatalysts
of this invention, the "droplets" are formed by the microspheres,
per se, or at least the liquid carrier medium present
therein. This feature provides another advantage of
the present invention, namely, that since all of the
microspheres are of uniform size, wall thickness, etc.,
all of the "droplets" are likewise of uniform size (diameter).
An additional advantage is that it is not necessary or
essential to form an emulsion in order to carry out the
interfacial polymerization.

In the encapsulation process of the above mentioned
patents to Lim and Moss a two step interfacial polymerization
process is provided. According to this procedure an
emulsion is formed wherein the discontinuous phase (droplets)
contains the substance (biocatalyst) to be encapsulated
and a first polymerizable monomer. A second monomer
which is reactive with the first monomer is added to
the continuous phase and an imperfect membrane forms
about the droplets of the discontinuous phase. The continuous
phase is removed to terminate the polymerization and
the partially encapsulated droplets are resuspended in
a liquid to which an additional amount of the second
monomer is added causing further polymerization, strengthening
the permselective membrane, and "patching" any microporous
defects.

The Lim and Moss encapsulation process is useful
where the biocatalyst is, for example, an enzyme, hemoglobin,
antibody, or other "non-living" biochemically active
substances. For encapsulation of living microorganisms,
cells, cell tissues, and the like the process of the
Lim patents or Sefton, for example, is preferred.

According to the above-mentioned patents to Lim
encapsulation of the core material (e.g. living tissue,

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individual cells, etc.) is carried out by suspending
the core material in an aqueous medium which contains
a water soluble gum that can be reversibly gelled to
form a coherent shape-retaining mass, forming the suspension
into droplets, changing the conditions of temperature,
pH or ionic conditions to cause gellation of the gum
to form a temporary protective barrier, and thereafter
forming a permanent semipermeable membrane by any suitable
technique about the temporary capsules.

Sefton encapsulates live animal cells in a polymeric
permselective membrane by first preparing an aqueous
suspension of viable cells and bringing the aqueous suspension
into contact with a solution of a bio-compatible, water-insoluble
polymer in a polar, non-toxic liquid, optionally also
in the presence of a water immiscible suspending medium,
and forming a suspension of aqueous droplets containing
the viable cells in a hydrophobic liquid medium continuous
phase. The polymer is caused to precipitate either by
addition to the mixture of an additional non-solvent
for the polymer or substantially spontaneously due to
the non-solvent character of the suspending medium.
The precipitated polymer forms the semipermeable membrane
encapsulating the cells in the droplets.

Any of the above or any other known encapsulating
technique can be used in the present invention. For
example, to apply the technique of Lim and Moss to the
present invention it is merely necessary to incorporate
the first monomer in the liquid carrier medium with the
biocatalyst. After the microspheres are filled with
the liquid medium - biocatalyst - monomer system the
filled microspheres are separated from the remaining
(external) liquid medium - biocatalyst - monomer system
and are resuspended in the same or different liquid medium
to which the second monomer is added. As soon as the
second monomer diffuses into the intracapsular space
via the entrance means and pores of the microspheres,
the first and second monomers will react to form a permselective
membrane sealing the pores and openings and thereby providing

a protective barrier for the biocatalyst contained within
the microsphere.

The technique of Lim can be applied to the filled
microspheres by incorporating in the liquid medium -
biocatalyst system the water soluble reversible gel forming
substance. Then, after transferring the filled microspheres
to a liquid medium not containing any of the gel forming
substance (or biocatalyst), the conditions in the media
are modified to cause gellation of the gel forming substance,
at least in the pores and openings in the walls of the
microspheres. This can conveniently be accomplished
by, for example, using a water-soluble gum having acid
groups as the reversible gel forming substance and adding
a solution of multivalent cations to the liquid medium.
As the multivalent cations diffuse through the entrance
means and pores in the walls of the microspheres and
contact the gum the gum is rendered water-insoluble,
i.e. is gelled, to effectively plug the pores and entrance
means and provide a temporary protective barrier for
the biocatalyst. Thereafter, a permanent selective membrane
is deposited, by any convenient technique which is not
detrimental to the biocatalyst, on the gel plugs. As
disclosed, for example, in the Lim U.S. Patent 4,352,883,
the permselective membrane may be formed by interfacial
polymerization using a pair of at least difunctional
mutually reactive monomers (as in the Lim and Moss U.S.
Patent 4,324,683), or one monomer and a relatively low
molecular weight polymer; or by interfacial polymerization
using polyaddition reactions; or by permanently cross-linking
surface layers of the gel by subjecting them to an aqueous
solution of a polymer containing groups reactive with
functionalities in the gel molecules.

The encapsulation technique disclosed by Sefton
can be adapted to the filled microspheres according to
the present invention by suspending the filled microspheres
in a polymer solution and adding to the suspension an
additional non-solvent for the polymer causing the polymer
to precipitate on the microspheres and form a permselective

membrane. Preferably, however, the permselective membrane
formation is limited to within the entrance means and
pores in the walls of the microspheres by including in
the liquid medium - biocatalyst system an additional
suspending medium which is a non-solvent for the polymer.
Then when the microspheres filled with the liquid medium-biocatalyst-
non-solvent suspending medium is contacted with the polymer
solution the polymer can diffuse into the entrance means
and pores of the wall and will precipitate as a thin
film as soon as it contacts the non-solvent suspending
medium.

     According to one preferred technique, a permanent
semi-permeable membrane material is formed across the
entrance means and pores by washing the gel protected
microspheres in an isotonic salt solution or in deionized
water and, thereafter, contacting the washed microspheres
with a semi-permeable film-forming material or a precursor
thereof, e.g. a prepolymer, oligomer, or monomer system
with polymerization catalyst and, optionally a cross-linking
agent. The gel may function merely as a support for
the polymer film in the pores and macropores or the gel
material at the gel surface and the film-forming material
may react with each other, such as by cross-linking,
to form the semi-permeable film.

     Any suitable method can be used for depositing
the semipermeable film. For instance, the microspheres
can be immersed in a dilute solution of a film-forming
polymer or a polymer forming system, preferably while
applying mild agitation to the mixture to ensure homogeneity
and uniform formation of the film on all of the microspheres.
It is also preferred to carry out the contact with a
small overpressure on the system to force the polymer
film into the pores and macropores, as well as between
and among the individual sintered particles of the microsphere
walls and defining the pores and macropores, this serving
to strengthen the bond between the formed membrane and
the microsphere. It should be noted that the application
of pressure to the film forming solution in which the

microspheres are immersed can be analogized to the application
of a vacuum to the bore of hollow porous filaments to
which a permselective polymer coating is applied from
the exterior, such as taught in U.S. Patents 4,230,463,
and 4,214,020. The coating process is generally suitable
for applying a depositable film forming material to form
a deposit of a semi-permeable membrane in the pores and
macropores of individual microspheres over essentially
the entire surface areas of the hollow microspheres. Any
potential for formation of air pockets is reduced or
any air pockets which do form can be dissipated by the
pressure exerted on the deposited material, as well as
by diffusion or permeation of the air pocket into the
gel, the surrounding interconnecting voids between the
wall forming particles, or through the deposited material
itself. The deposited films are sufficiently thin to
provide high flux rates of gases, liquids and solutes,
having molecular weights below the "cut-off" weight
of the deposited semi-permeable film. The "cut-off" weight
refers to the maximum molecular weight of dissolved solutes
which can diffuse through the semi-permeable membrane.

In further detail, the depositable material,
i.e., a material suitable for forming the coating, desirably
has a sufficiently large molecular size (if dissolved
in the solvent) or a sufficiently large particle size
(if suspended in the solvent, say, as a colloidal suspension)
that the depositable material does not readily pass through
the gel-filled pores in the walls of the hollow microspheres
when subjected to pressure. Thus, with microspheres
having pores of generally larger diameters, depositable
materials which have larger sizes when in the coating
liquid are frequently desired. In some instance, it
is desirable to employ depositable materials which, when
in the liquid substance, have sufficiently small sizes
that they can enter, instead of bridge, pores in the
microspheres. The depositable material may directly
form the coating when deposited, or the deposit of the
depositable material may be further treated, e.g. by

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cross-linking, to form the desired coating.

The coating liquid generally comprises a solvent
(or vehicle) for the depositable material. The solvent
should be capable of dissolving the depositable material
or be capable of enabling a finely-divided suspension
of the depositable material, say, having particle diameters
less than about 1 micron, e.g. about 5000 angstroms (i.e.,
colloidal size) to be provided. Desirably, the coating
liquid contains substantial amounts of solvent, e.g.
a major amount of solvent, such that during deposition
of the depositable material on the hollow microsphere,
depositable material, which is not forcibly retained
in the pores and macropores due to adhesion to the gel
and /or solid wall material and/or due to the presence
of the applied pressure, can be redissolved or otherwise
removed from the vicinity of the hollow microspheres.
Also, the coating liquid contains sufficient amounts
of solvent such that the coating liquid exhibits a viscosity
at temperatures employed in the coating which viscosity
is advantageously low to enable relatively rapid, adequate
permeation of the coating liquid through a dense mass
of the microspheres. Often, the viscosity of the coating
liquid at such temperatures is less than about 25 or
50 centipoise, and in some instances less than about
10 centipoise, say, about 0.1 to 5 centipoise. Often,
the depositable material comprises less than about 20,
preferably, less than about 15, say about 0.1 to 15,
weight percent of the coating liquid. Most frequently,
the depositable material comprises 0.5 to 10, weight
percent of the coating liquid.

The contact of the coating liquid containing
the depositable material with the mass of microspheres
in order to effect the desired deposition is advantageously
provided by immersion of the mass in the coating liquid.
The immersion of the hollow microspheres in the coating
liquid may be effected in any suitable manner. For instance,
the mass of microspheres may be poured into the coating
liquid. However, it is generally preferred that the

coating liquid be added to a retaining vessel containing
the mass of microspheres.

The coating liquid may be unagitated or may preferably
be agitated, e.g. by circulating the coating liquid through
the retaining vessel to assist in maintaining the suspension
of the depositable material (if the depositable material
is in particulate form in the coating liquid) and in
providing desirable distribution of the coating liquid
through the microsphere mass.

The coating liquid may be at any suitable temperature
immediately before and during the immersion which is
not deleterious to the coating liquid, the gel, biocatalyst
or the hollow microspheres. The temperature should be
sufficiently high to provide the coating liquid with
a desirable viscosity for conducting coating but not
unduly elevated such that either the coating liquid,
particularly the depositable material, or the biocatalyst,
etc., are adversely affected. The temperatures of the
coating liquid immediately before and during the immersion
are below the boiling point of the coating liquid under
the conditions of coating operation, and preferably are
within the range of about -10° to 70°C, say about 0°
to 50°, and most conveniently, about ambient temperature,
e.g. about 5° to 40°C.

The hollow microspheres are subjected to the
applied pressure from the exterior to the interior at
least while immersed in the coating liquid. The pressure
drop is desirably maintained for a time (either intermittent
or preferably continuous) sufficient to provide the desired
deposit. The pressure drop from the exterior to the
interior during the immersion is sufficient to provide
the desired deposit of depositable material. Generally,
the pressure is at least about 5 pounds per square inch
gauge, say, about 5 to 100 or more, pounds per square
inch gauge. Although higher pressure drops can be employed,
such high pressure drops are not necessary, since often,
little, if any, benefit is obtained by increasing the
pressure to greater than about 50 pounds per square inch.

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Preferably, the pressure is about 10 to 50 pounds per square inch. The pressure drop is often maintained for a duration of at least about 0.01 hour, e.g., about 0.05 to 10 or 50 hours, say about 0.05 to 5 hours.

The microspheres are immersed in the coating liquid for a sufficient duration to provide the desired deposition of the depositable material. Generally, the duration of immersion depends on the mass of the microspheres, the packing density of the hollow microspheres, the viscosity of the coating liquid, and the applied pressure. In many instances, the duration of the immersion will be at least about 0.01 hour and may be up to about 50 or more hours, e.g. about 0.05 to 10 hours, say about 0.05 to 5 hours.

Since the gel may often extend to the outer surface of the microsphere wall, that is, completely fill the interstitial spaces between the sintered together particles forming the wall, as well as the macropore volume extending through the wall, it is often preferred in order to increase the bonding or anchoring of the depositable material forming the permselective membrane to the sintered together particles, to displace a portion of the gel at and below the outer wall surface to expose the sintered together particles. This may be accomplished, for example, by reliquefying the outer portion only of the gel, for example, by the technique described below, or by slightly raising the temperature of the system, and thereafter washing out the reliquefied gel before depositing the membrane material. Another method involves adding minute gas bubbles to the system, for example, by application of high frequency agitation to cause cavitation at the surface of the microspheres and displacement of the gel by the bubbles. The agitation may be sonic or acoustic. The amplitude of the vibration will be selected depending on the desired depth of displacement but generally can be about one-eight to one-fourth or more of the thickness of the microsphere wall. Furthermore, if the intracapsular space is not completely filled with the gel material

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a portion of the gel present in the microsphere wall
may be displaceable into the intracapsular space by applica-
tion of fluid pressure to push the gel out of the interstitial
spaces (interconnecting voids) into the intracapsular space.

The emersion of the coated microspheres may be
by removing the microspheres from the coating liquid
or by preferably draining the coating liquid from the
retaining vessel.

After the emersion of the coated microspheres
from the coating liquid, the microspheres may be immersed
in at least one additional coating liquid, which additional
coating liquid may or may not be essentially the same
as that of the first coating liquid, in order to provide
two or more coatings on the hollow microspheres or to
chemically affect at least one preceding deposit, e.g.,
by cross-linking or the like. Cross-linking or other
procedures which chemically affect the deposit may be
provided by contact with a suitable liquid or gaseous
agent. The deposits on the hollow microspheres may or
may not be dried (i.e. the remaining solvent removed)
or otherwise treated intermediate the immersions.

Frequently, after the emersion from the coating
liquid, the deposit is dried, i.e., the remaining solvent
is removed. The drying can be conducted in any suitable
manner. For instance, the microspheres may be air dried
or gases which are unsaturated with the solvent may be
passed through the mass of the coated microspheres. Preferably,
in instances in which the solvent poses a fire risk,
the drying gases are substantially inert such as nitrogen,
carbon dioxide, etc. The temperature of the drying gases
may vary substantially provided that the desired drying
occurs and the material deposited and the encapsulated
biocatalyst are not unduly adversely affected. For the
sake of convenience, the drying gases may be at slightly
elevated temperatures, e.g. about 30° to 70°C. The drying
is generally conducted for a time sufficient to remove
substantially all of the solvent from the coating, e.g.
often about 1 to 48 hours. After drying, the deposit

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on the exterior surfaces of the hollow microspheres is
sufficiently nontacky that no undue sticking of the hollow
microspheres occurs.

The depositable material which is deposited may
not directly form the desired coating but rather may
be a precursor for the coating. Thus, the depositable
material may be capable of reacting with one or more
simultaneously or subsequently provided components, or
it may be capable of homopolymerizing, to provide the
desired coating. Therefore, conditions are provided
during the drying and/or subsequent to the drying to
chemically convert the depositable material to the desired
material of the coating. Hence, coatings can be provided
that are comprised of materials which materials would
not be desirable as depositable materials.

The resulting coating (or coatings) are relatively
uniform throughout all the pores and interconnecting
voids and macropores. Generally, the coating has an
average thickness of up to about the wall thickness,
preferably about 1/2 to 1/50 of the wall thickness.
Frequently, the average thickness of the coating is less
than about 5, and may even be about 1 micron or less.
Advantageously, the coating is substantially permanent
in the pores and interconnecting voids and macropores
and thus does not unduly separate from the hollow microsphere
during storage or use of the bioreactors in biotech operations.

The hollow microspheres prior to being coated
are porous, i.e. have continuous channels for fluid flow
extending between the exterior and interior surfaces.
Frequently, the pores have an average cross-sectional
diameter of from about 5000 to about 40,000 angstroms
(0.5 to 4 microns) and in some hollow microspheres, the
cross-sectional diameter of the pores or interconnecting
voids may be less than about 1000 or more than about
50,000 angstroms.

Passage across the semipermeable membrane may
be by chemical or molecular diffusion and/or by solvation
and evaporation and is dependent on the molecular spacing

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of the coating material.

In a preferred embodiment for carrying out the encapsulation of the biocatalyst, especially living cells and microorganisms (hereinafter jointly referred to for convenience as "cells" or "cellular biocatalyst,") in the microsphere container, the following sequence of steps is carried out:

1.   A homogeneous suspension of the cellular biocatalyst in a solution of a long-chain polyanion gel former is prepared.

2.   The macropore containing microspheres are filled with the cellular biocatalyst suspension using hydrostatic pressure, centrifugal forces, etc.

3.   The filled microspheres are contacted with a physically compatible multivalent ion to replace the monovalent ion of the gel former to cross-link the latter and form a temporary discontinuous gel membrane.

4.   A permanent membrane is deposited on the gel membrane surface by appropriate reaction and polymerization.

5.   The temporary gel membrane is removed by contact with a monovalent ion solution leaving intact the permanent permselective membrane.

These steps will be described in greater detail below.

Step 1. Biocatalyst Suspension

Healthy cells with a viability of greater than 95% are first collected by centrifugation. The cell pellet is then resuspended in the presence of additional components, which are defined as those specific protein or other components which need to remain closely associated with the cells to either promote growth or aid in the production and secretion of the specific macromolecules.

The nutrient medium employed will be dependent upon the cellular biocatalyst involved and the product desired or purpose for the reactor. For example, the nature of the nutrient medium will be adapted to the

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particular type of cells or microbes. Besides nutrients,
other substances may be included to support growth and/or
cell differentiation or to provide a particular product.

The cells are incubated in a nutrient cell culture
medium under cell growth maintenance conditions of pH
and temperature. Suitable nutrient cell culture media
are known to the art and such may be used in the method
of the present invention. Typically such nutrient culture
media contain the known essential amino acids, vitamins,
carbohydrates, mineral salts and, preferably, blood serum.
Fungicides and bacteriacides may also be included in
such media in desired amounts to prevent the growth of
undesired microorganisms. As indicated above the pH
of the nutrient medium is advantageously controlled within
the desired range (typically in the range of 6.8-8.2)
by including small amounts of carbon dioxide in the oxygen
carrier. However, if desired the pH can be controlled
by including a suitable buffer such as HEPES buffer (a
mixture of N-2-hydroxyethyl piperazine and N'-2-ethane
sulfonic acid) in the nutrient cell culture medium itself.
Other suitable methods for controlling pH such as passing
the medium over ionic exchange resins may also be employed.
The resulting cell slurry is then combined with 1.2-1.4%
(v/v) solution of alginic acid or other gel-former in
isotonic saline.

Step 2. Filling microspheres (Droplet Formation)

The macropore containing hollow rigid microspheres
(preferably formed from inorganic solid particles) prepared
in accordance with the techniques disclosed in the aforementioned
application LBT-32 are loaded into one or more centrifuge
tubes and the cell slurry prepared in step 1 is added
to the tube or tubes. The diameter of the macropores
will be selected to be at least 2 times, preferably from
about 2 to 5 or more times the major dimension of the
cellular biocatalyst.

The centrifuge tube is rotated at a rate sufficient
to provide dynamic pressures within the ranges described
above, until the microspheres are filled with a liquid

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"microdroplet" containing the viable cellular biocatalyst
distributed homogeneously throughout the microdroplet
at a density determined by the initial ratio of cells
to sodium alginate.

Step 3.   Gel Formation
        The rigid microsphere housed liquid microdroplets
are recovered from the centrifuge tubes and are added
to a solution of divalent or multivalent cations. Calcium
ions are the preferred divalent cations for gelling sodium
alginate. A calcium alginate gel which is shape-retaining
is formed in at least the pores and macropores and generally,
also in its interior, the liquid microdroplet is converted
to a shape-retaining fluid permeable gel which is harmless
to the entrapped cellular biocatalyst.

Step 4.   Semipermeable Membrane Formation
        The gel entrapped cellular biocatalyst protected
by the rigid microsphere containers are next contacted
with a saline solution containing a long-chain polycation.
Some of the long-chain polycations used in this membrane
formation step include polymers of the L-amino acids,
lysine, argine, ornithine, polymers of the D-amino acid
lysine, and synthetic organic polymers such as polyethyleneimine
and polyvinylamine. Interaction of the polycation with
the surface of the calcium alginate gel results in the
formation of a tight mixed-gel membrane which has defined
permeability characteristics.
        Immediately following formation of the semipermeable
membrane, the resulting microsphere bioreactors are washed
with a saline solution containing a dilute concentration
of a long-chain polyanion. This reaction neutralizes
the high positive charge density imparted to the surface
of the membrane due to the presence of the coating polycation.
Following this step, the membranes display a net neutral
surface charge behavior.

Step 5.   Liquefication of the Microdroplet Interior
        Following the deposition of the membrane at the

surface of the calcium alginate gel, the resulting bioreactor
is treated with a chelating agent capable of sequestering
the gel-catalyzing cation, but not capable of disrupting
the interaction between the long-chain polycation and
the surface alginic acid. The resulting chelation, in
the presence of excess sodium ions, results in conversion
of the calcium alginate gel to sodium alginate. Depending
on the molecular weight of the sodium alginate used in
the initial cell suspension, a predetermined percentage
of the sodium alginate diffuses from the intracapsular
space to the extracapsular space, thereby decreasing
the absolute intracapsular concentration of alginic acid,
and leaving behind the "permanent" semipermeable membrane.

The parameters which affect the permeability
of the semipermeable membrane include the following:

1. The concentration of the coating polycation.
2. The molecular weight of the coating polycation.
3. The secondary structure of the coating polycation.
4. The duration of the reaction.
5. The temperature under which the reaction
    takes place.

The permeability of the microcapsule membrane
is generally expressed in terms of the molecular weight
of globular polypeptides. Using this system of measurement,
membrane permeabilities can be obtained in the range
of <50,000 daltons to > 900,000 daltons. A preferred
molecular weight cut-off limit is about 300,000 daltons,
especially about 200,000 daltons.

Following liquefaction, the bioreactors will
be washed several times with a clear saline solution.
The bioreactors are then ready for use in a biotech process.

Naturally, other techniques for forming the permselective
membrane at least in and across the entrance means and
pores/channels in the walls of the microspheres can be
used, so long as the permselective membrane can be formed
and/or deposited under conditions which are nondetrimental
to the continuing viability or activity of the biocatalyst.

Advantages

The permselective membrane coated microspheres
provide a sterile nutrient environment for the biocatalyst
contained therein. In comparison to the bioreactors
formed by encapsulating biocatalysts in polymeric microspheres,
such as described in the above-mentioned patents to Lim,
Lim and Moss or the patent to Sefton, wherein the polymer
walls provide the joint function of permselective membrane
and support structure, the bioreactors of the present
invention have the important advantage of uncoupling
the structural support function from the selective separation
function. Accordingly, the permselective membranes can
be much thinner than the wall thickness of the prior
art microcapsules with correspondingly higher flux rates,
while still providing much greater overall strength.
For example, the permselective membrane coated microspheres
of this invention can withstand hydrostatic pressure
up to at least about 1500 psi, and two point pressures
up to at least about 200 psi. In view of this high wall
strength, the bioreactors are much more easily handleable
and transferable and can be used in fixed bed processes
at high packing densities, for example, bed heights of
up to 30 feet or more, and in fluidized bed processes
wherein bioreactor-to-bioreactor contact or bioreactor-to-tower
wall impacts even at high speeds do not cause any structural
damage to the microsphere walls or stress to the permselective
membranes. Furthermore, the generally low density and
mass of the microsphere containers contributes to this
advantage and also reduces the shear and impact forces
which could be harmful to certain biocatalysts.

Because of their exceedingly low cost, the permselective
membrane coated microspheres can be discarded periodically
for short lifetime membrane materials or they can be
recycled for regeneration of the permselective membrane
and/or for renewal and/or collection of the biocatalyst
and/or collection of product.

In addition, the bioreactors of this invention
are much more uniform in size than the prior art microcapsule

bioreactors. Furthermore, a much wider range of diameters
and wall thicknesses are available for the hollow porous
rigid microsphere containers than for the liquid microdroplets
used in the conventional encapsulating techniques. Therefore,
control of process parameters, e.g. mass flow rates,
fluid dynamics, heat transfer, etc., is greatly simplified.

Because the microspheres are hollow and have
porous walls, they will generally have bulk densities
which are significantly lower than the density of the
solid particles forming the microsphere walls, e.g. in
the range of from about 0.02 g/cm^3 to about 2.4 g/cm^3,
preferably from about 0.03 g/cm^3 to about 1 g/cm^3, and,
therefore, when filled will approximate the density of
the aqueous systems used in most biotech processes, and
therefore, the bioreactors, i.e. microsphere encapsulated
biocatalysts, will be buoyant or at least easily suspended
in the aqueous culture broth or other liquid medium,
e.g. aqueous waste stream, etc., used in the biotech
process. The bulk density of the bioreactors will not
be significantly increased by the permselective membrane
since the material of the permselective membrane will
generally have a lower density than that of the solid
particles, and/or because the permselective membranes
are generally substantially thinner than the microsphere
wall thickness.

Still, another advantage of the bioreactors of
this invention is that the microsphere containers, while
non-deformable under conditions of use in the biotech
processes, can be broken where necessary or desired for
removal and/or recovery of the biocatalyst and/or products,
etc. The microsphere wall will absorb substantially
the entire amount of pressure exerted to effect the rupture
of the wall without adversely effecting the biocatalyst
and/or product. In contrast, prior art microcapsules
which have relatively soft and deformable walls will require
very high pressures to cause rupture and will also transmit
these high pressures to their contents.

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Furthermore, the preceding advantages apply equally to the gel-immobilized biocatalyst encapsulated within the rigid microsphere wall structure.

Biotech Processes

As used herein the term "biotech" process is given as broad a meaning as possible consistent with the requirement that it involves the use of at least one reactant or substrate which is modified, converted, altered, concentrated, adsorbed, desorbed, or otherwise reacted, more or less specifically, through the assistance of a biocatalyst in order to manufacture, purify, separate, identify or quantify particular chemical and biological substances.

Thus, biotech processes encompass such diverse technologies as fermentation processes, biological waste water treatment, cell culture techniques, enzyme and bacterial catalyzed biochemical reactions, protein binding assays, drug delivery systems, affinity chromatography, and the like. Cell culture techniques include both growth of cells into cell tissues or cell masses for use as such, as well as growth and/or maintenance of cells for production of cell products such as hormones, enzymes, amino acids, nucleic acids, nucleotides, nucleosides, phosphatides, glucosides, purines, interferons, lymphokines, growth factors, and other biological substances.

Virtually any of the existing biotech processes can be adapted to advantageously employ the bioreactors of the present invention, regardless of the nature of the biocatalyst.

Example 1

This is an example of a fermentation process utilizing the novel bioreactors of this invention for the production of ethanol from fermentative sugar, e.g. glucose, such as by the process described in U.S. Patent 4,350,765 to Chibata, et al using immobilized ethanol-producing microorganisms of the genera Saccharomyces

and <u>Zymomonas</u>. The hollow porous alumina microspheres
of Example 4 of the aforementioned application Serial
No. 639,126 having an outer diameter of about 2500 microns
(2.5 mm) and a wall thickness of about 20 microns, with
entrance means (macropores) of about 25 microns in diameter
extending through the wall thickness, and an average
pore diameter of from about 0.1 to 3 microns are used
as the bioreactor container. To fill the hollow microspheres
with the microbes, a suspension of the microbes, for
example, <u>Sacch.</u> <u>sake</u> ATCC 26422 (Japan strain Kyokai
7), in a gel forming carrier, such as a sterilized 4.5
w/v% aqueous solution of carragenan (manufactured by
the Copenhagen Pectin Factory, Ltd. under the tradename
"GENUGEL Type WG") at a concentration of about one loopful
per 20 ml is placed in a centrifuge tube maintained at
about 37°C. The hollow microspheres, after sterilization
if necessary, in an amount of about 50% to about 65%
by bulk volume based on the volume of the gel forming
carrier, are added to the centrifuge tube and the mixture
is centrifuged at about 5 psig for about 200 minutes.
The excess gel forming carrier is decanted off from the
filled microspheres and the filled microspheres are then
added to an about 2 w/v% aqueous solution of potassium
chloride whereby a gel forms in at least the entrance
means and pores to seal in the microbes.

     After washing the microspheres with the gel entrapped
yeast contained therein with a sterile aqueous saline
solution, the bioreactors are ready for use.

     A nutrient broth is prepared by admixing a yeast
extract (0.15 w/v%), ammonium chloride (0.25 w/v%), dipotassium
hydrogenphosphate (0.55 w/v%), magnesium sulfate heptahydrate
(0.025 w/v%), calcium chloride (0.001 w/v%) citric acid
(0.1 w/v%) and sodium chloride (0.25 w/v%) in water and
adjusting the pH thereof to 5.0.

The bioreactor containing the gel-immobilized
yeast (20 ml) is contacted with the nutrient broth containing
10 w/v% of glucose (20 ml) at 30°C for 1 hour. When
the concentration of the remaining glucose in the broth
is lowered to 2 mg/ml, the additional fresh nutrient
broth containing 40 w/v% of glucose (5 ml) is added and
the conversion reaction of glucose into ethanol is continued
for another 1 hour under the same condition. Thereafter,
the addition of the nutrient broth containing 40 w/v%
of glucose (5 ml) is repeated 3 times at an hour-intervals.
As a result, the immobilized yeast keeps its ethanol-producing
activity at the level of 50 mg/ml of the gels/hr during
the reaction period and the broth containing ethanol
in a concentration of 125 mg/ml (40 ml) is obtained after
the conversion reaction for 5 hours.

Example 2

Using the same microsphere bioreactors as produced
in Example 1, ethanol production is carried out using
a cylindrical columnar reactor. A column having a circulating
tube which is able to circulate a portion of a broth
flowed through column 1 to the bottom of the column is
prepared by packing the bioreactors (2.5 liters) into
the column, feeding 20 w/v% of an aqueous molasses solution
(100 mg/ml as glucose) to the bottom of the column through
a broth feeding pipe at the rate of 2.5 liter/hr at 30°C
to float the bioreactors in the column and flowing out
the broth from the top of the column through an outlet
pipe at the same rate. After incubation at 30°C for
40 hours while feeding the broth at the above feed rate,
50 w/v% of the aqueous molasses solution (250 mg/ml as
glucose) is fed to the column at a rate of 1 liter/hr
while a portion of the broth flowed through the column
is circulated to the bottom of the column through the
tube circulating at a rate of 10 liter/hr. The concentration
of the aqueous molasses circulating through the tube
is lowered to not more than 10 mg/ml as glucose. Under
this condition, ethanol producing activity of the immobilized.

yeast is not lowered and an effluent containing ethanol
in the concentration of 125 mg/ml is continuously flowed
out from the outlet pipe at the top of the column since
the concentration of the aqueous molasses in the broth
to be fed to the column is diluted with the circulated
broth through the tube.

Example 3

Islets of Langerhans obtained from rat pancrease
are added to a complete tissue culture (CMRL - 1969 Connaught
Laboratories, Toronto, Canada) at a concentration of
approximately 10^3 islets per milliliter. The tissue
culture contains all nutrients needed for continued viability
of the islets as well as the amino acids employed by
the Beta cells for making insulin. Four milliliters
of the islet suspension are then added to five milliliters
volume of 1.2 percent sodium alginate (Sigma Chemical
Company) in physiological saline.

Following the teachings of the hollow porous
microsphere manufacturing process of application Serial
No. 639,126, microspheres having a diameter of about
5000 microns and a wall thickness of about 100 microns
and containing in their walls a plurality of macropores
of about 200 microns, are prepared.

Two 20 ml centrifuge tubes are each filled with
4 ml of the hollow microsphere containers and the culture-
medium tissue suspension in two equal portions of 4.5
ml each are added to the centrifuge tubes. The tubes
are rotated at 600 rpm for 45 seconds and the excess
culture-medium tissue suspension is decanted off and
the culture-medium tissue suspension filled microsphere
are recovered.

Next 800 milliliters of a 1.5% $CaCl_2$ solution
are placed in a 1.5 liter beaker equipt with a stirrer
and the hollow porous rigid microspheres filled with
the culture-medium tissue suspension are slowly introduced

into the CaCl₂ solution whereupon the sodium alginate
in the macropores and smaller pores immediately begins
to gel.

After 10 minutes, the stirrer is turned off and
the supernatant solution is removed by aspiration. The
microspheres are then transferred to a beaker containing
150 ml of solution comprising one part of a 2% 2(cyclohexylamino)
ethane sulfonic acid solution in 0.6% NaCl (isotonic,
pH=8.2) diluted with 20 parts 1% CaCl₂. After a 3 minute
immersion, the microspheres are washed twice in 1% CaCl₂.

The microspheres are then transferred to a solution
comprising 1/80 of one percent polylysine (average MW
35,000 AMU) in physiological saline. After 3 minutes,
the polyslysine solution is decanted. The microspheres
are then washed with 1% CaCl₂, and then suspended for
3 minutes in a solution of polyethyleneimine (MW 40,000-60,000)
produced by diluting a stock 3.0% polyethyleneimine solution
in morpholino propane sulfonic acid buffer (0.2 M, pH=6)
with sufficient 1% CaCl₂ to result in a final polymer
concentration of 0.10%. The resulting microspheres have
permanent semipermeable membranes extending across the
macropores and pores at the exterior wall surface. The
resulting bioreactors are then washed twice with 1% CaCl₂,
twice with physiological saline, and mixed with 100 ml
of a 0.12 percent alginic acid solution.

The microspheres resist clumping and contain
islets of Langerhans. Gel on the interior of the capsules
is reliquified by immersing the microspheres in a mixture
of saline and critrate buffer (pH=7.4) for 5 minutes.
Lastly, the capsules are suspended in CMLR - 69 medium.

Under the microscope, the microcapsules have
an appearance illustrated in the Fig. 4. They comprise
an ultrathin membrane 44 extending within the macropores
and pores near the outer periphery of the microsphere
wall. One or more islets 54 each consisting of many
individual cells are suspended in the liquid culture

medium 55 in the intracapsular space 50 and are protected
against attack by bacteria, viruses and other microbes.
However, molecules having a molecular weight up to about
100 thousand can traverse membrane 44. This allows oxygen,
amino acids, nutrients, and plasma components used in
culture media (e.g. fetal calf plasma components) to
reach the islet and·allows insulin to be excreted.

Similar results to the above are achieved with
other cellular biocatalysts, for example, red blood cells
using serum as a medium, sperm cells, using semen as
the medium, and baker's yeast.

Cell cultures encapsulated as described above
may be suspended in culture media designed specifically
to satisfy all of the requirements of the particular
cell type involved and will continue to undergo normal
in vitro metabolism. If the culture requires an environment
of high molecular weight components such as serum components,
these may be omitted from the extracapsular medium. Typically,
the components normally ingested by cells are of relatively
low molecular weight and readily diffuse across the capsule
membranes into the microenvironment of the cells where
they permeate the cell membrane. Products of metabolism
of the cells which are secreted into the intracapsular
medium, if they have a molecular weight below the upper
limit of permeability of the capsule membrane, likewise
diffuse thereacross and collect in the extracapsular
medium.

The encapsulated cells may be cultured under
conditions of, e.g. temperature, pH and ionic environment,
identical to conventional cultures. Also, cell-produced
products may be harvested from the extracapsular medium
or from capsules by conventional techniques. However,
the culturing technique.disclosed herein has the following
advantages:

1. The cells of the culture are protected from
contamination by factors having dimensions in excess

of the upper permeability limit of the membranes. This
means that handling and sterility requirements normally
incident to culturing procedures can be somewhat relaxed,
since microorganisms cannot reach the encapsulated cells,
and virus infected cells need not contaminate other cells.

2. The microcapsules in effect immobilize the
cells within an environment in which enclosed high molecular
weight materials are confined, yet lower molecular weight
cell nutrients and products are readily removed and introduced.
This allows the nutrient broth to be intermittently or
continuously collected and supplemented as desired, without
disturbing the cells.

3. Substances of interest produced by the cells
are more easily recovered. Secreted cell products of
molecular dimensions small enough to permeate semipermeable
membranes collect in the extracapsular medium in admixture
with nutrients. However, high molecular weight serum
components and the like are not released into the extracapsular
medium, thus simplifying recovery of a cell product of
interest. Secreted cell products of molecular dimensions
in exces of the upper permeability limit of the membranes
collect within the capsules. Of course, cell products
not secreted through the cell membrane may also be of
interest. These may be recovered in relatively concentrated
form by isolating the microspheres and subsequently selectively
disrupting the membranes using, for example, the technique
disclosed hereinafter, and if necessary by disrupting
the cell membranes.

4. The intracapsular volume provides an environment
well suited for cell division. Suspension cultures undergo
mitosis within the microspheres. Anchorage dependent
cells which in normal cultures grow in a two-dimensional
monolayer multiply to form an array within the microsphere.
Such cells use the interior surfaces of the membrane
as a substrate and/or anchor to the solid particulate
wall forming materials set forth above. This leads to

significant increases in cell density as compared with
conventional cultures. The ongoing viability of such
cell clusters is aided by the fact that the surface area
to volume ratios of the capsules can be quite large,
and thus, all cells have access to required nutrients,
oxygen, etc.

     5. The solid rigid walls provide all the required
structural support for and protect the semipermeable
membrane from shearing forces and mechanical damage. Therefore,
the semipermeable membranes can be substantially thinner
than in conventional semipermeable membrane microcapsules
wherein the membranes also provide the structural support.
This allows the bioreactors of this invention to be used
at much larger packing densities, in long columns, in
high rate continuous flow and fluidized bed processes
and the like.

     In certain situations, it would be advantageous
to selectively disrupt the membranes to release the cells
without damage. One notable example is in the production
of interferon (INF). Cells capable of producing INF
must be subjected to certain viruses or nucleic acids
in preparation for the INF production stage. Also, in
several INF induction procedures, reagents are added
to the culture to inhibit protein synthesis. Accordingly,
the growth stage of the culturing process must be conducted
under conditions quite different from the INF induction
stage. If the substances used for INF induction are
of a molecular weight in excess of the upper permeability
limit of the capsule membranes (as will be the case in
virus inductions), the induction process cannot be accomplished
in the encapsulated cell culture. Accordingly, IFN producing
cells, if grown within the microcapsules, would have
to be released by disruption of the membrane in order
to be subjected to the induction process.

     Cells confined in microspheres sealed with semi-
permeable membranes of the type set forth above may be

released by a process involving commercially available
reagents having properties which do not significantly
adversely affect the encapsulated cells. First, the
microspheres are separated from their suspending medium,
washed thoroughly to remove any contaminants present
on the exterior of the microspheres, and then dispersed
with agitation, in a mixed solution of monatomic, multivalent
cations, such as calcium ions and a stripping polymer
having plural anionic moieties, such as a salt of a polysulfonic
or polyphosphoric acid. Heparin, a natural sulfonated
polysaccharide, is preferred for this step. The anionic
charge density of the stripping polymer used should be
equal to or preferably greater than the charge density
of the polyanionic material originally employed to form
the membranes. The molecular weight of the polymer should
be at least comparable to and preferably greater than
the molecular weight of the polymer having plural cationic
groups used in forming the membrane. Within the suspension
of microspheres in the mixed solution, the calcium ions
compete with the polycationic polymer chains used to
form the membrane for anionic sites on the water-soluble
gum. Simultaneously, the heparin or other polymer having
plural anionic moieties dissolved in the solution competes
with the gum in the membrane for cationic sites of the
polymer chains. This results in a water-dispersable
or preferably water-soluble complex of e.g. polylysine
and heparin, and an association of the monatomic cations
with molecules of the gel.

This step renders the membrane susceptible to
dissolution upon subsequent exposure to a sequestering
agent which completes the disruption process by taking
up monatomic ions from the gel. Capsule membrane debris
which remains in the medium, if any, can be easily separated
from the cells which are allowed to diffuse or flow with
the medium through the now unblocked macropores to the
extracapsular space.

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The currently preferred solution for the first
stage of the selective disruption process comprises 1.1%
calcium chloride (w/v) and between 500 to 1,500 units
of heparin per milliliter of solution. A volume of microspheres
is added to this solution sufficient to constitute between
about 20% and 30% of the total volume of suspension.
Calcium chloride and heparin are preferred for disrupting
membranes of cell-containing microspheres since both
reagents are physiologically compatible with most cells
and, therefore, minimize the possibility of cell damage.
Mixtures of aluminum salts or other multivalent cations
(but not Mg++ ions) may also be used together with the
polysulfonic or polyphosphoric acid salts of the type
set forth above.

In general, the concentration of monatomic ions
and anionic polymer used in this step may vary widely.
Optimum concentrations may be readily determined empirically,
and depend on exposure time as well as the particular
polymer used to form the membranes.

The currently preferred sequestering agent for
performing the selective disruption is sodium citrate,
although other alkali metal citrate salts and alkali
metal EDTA salts may also be used. When sodium citrate
is employed, the optimum concentration is on the order
of 55 mM. It is preferred to dissolve the citrate or
other sequestering agent in isotonic saline so as to
minimize cell damage.

Example 4

One and one-half ml of an aqueous carrier solution
comprising polyvinylpyrrolidone, albumin, and 250 µl
of antisera to thyroxine are mixed with 50 µl of 0.5
M tetraethylene pentamine carbonate (pH=8.2-8.6). The
aqueous system is mixed by means of a magnetic stirring
bar, and as stirring continues, the mixture is added
to a pressure vessel containing 15 ml of the hollow porous

rigid microspheres used in Example 1 and the pressure
is increased to about 150 psi over a period of 60 minutes
and is held at 150 psi for an additional 30 minutes.
The pressure is then gradually released over a ninety
minute period to atmospheric pressure whereupon the excess
emulsion is decanted and the filled microsphere are washed
3 times with distilled water. One 20 ml portion of a
4:1 (v/v) cyclohexanechloroform solution containing 0.1
mg/ml terephthaloyl chloride is added to the filled microspheres
to initiate polymerization between the tetraethylene
pentamine carbonate and terephthaloyl chloride. Sixty
seconds later, another 8 ml of the terephthaloyl chloride
solution is added. After a total reaction time of four
minutes, the suspension is gently centrifuged and the
supernatant liquid discarded. The microspheres are washed
with pure cyclohexane and a 50% aqueous TWEEN-20 solution
(sorbitan monolaurate) buffered to neutral pH with 0.3
M $NaHCO_3$.

The foregoing procedure results in bioreactors
with encapsulated thyroxine antisera having a pore size
large enough to allow free passage of thyroxin, which
has a molecular weight of about 777 daltons, yet too
small to allow leakage of antibody from the interior
of the bioreactors.

WHAT IS CLAIMED IS:

1.   A bioreactor comprising
        a hollow, porous, non-rupturable microsphere
container, said microsphere having a single hollow central
void forming intracapsular space and a substantially
uniformly thick wall surrounding said intracapsular
space, said wall including entrance means through which
biocatalyst can be introduced into the intracapsular
space, biocatalyst within the intracapsular space and
fluid permeable means for immobilizing the biocatalyst
within the intracapsular space, said entrance means being
larger than the maximum dimension of said biocatalyst.

2.   The bioreactor of claim 1 wherein said microsphere
has an outer diameter in the range of from about 200
to 10000 microns and a wall thickness of about 1 to 1000
microns, the wall of said microsphere having a porosity
of from about 5 to 45%.

3.   The bioreactor of claim 1 wherein the ratio of wall
thickness to outer diameter is in the range of from about
1:4 to 1:500.

4.   The bioreactor of claim 1 wherein the microsphere
diameter is in the range of from about 1200 to 6000 microns
and the wall thickness is from about 10 to 200 microns.

5.   The bioreactor of claim 4 wherein the ratio of wall
thickness to outer diameter is in the range of from about
1:10 to 1:300.

6.   The bioreactor of claim 1 wherein the microsphere
wall is comprised of sintered together inorganic particles.

7.   The bioreactor of claim 6 wherein the inorganic particles
have a particle size in the range of from about 0.5 to
10 microns.

8.   The bioreactor of claim 7 wherein the inorganic particles are selected from the group consisting of metals, ceramics, glass, and glass-metals, and mixtures thereof.

9.   The bioreactor of claim 6 wherein the microsphere container is rigid.

10.   The bioreactor of claim 1 wherein the microsphere container is rigid.

11.   The bioreactor 1 wherein the microsphere container can withstand hydrostatic pressures of about at least 750 psi and two point contact pressures of about at least 100 psi.

12.   The bioreactor of claim 1 wherein said biocatalyst is at least one member selected from the group consisting of enzymes, hormones, antibodies, hemoglobin, interferon, and lymphokines, and mixtures thereof.

13.   The bioreactor of claim 10 wherein said entrance means is comprised of the pores in the microsphere wall, said pores having an average diameter in the range of from about 0.1 to 3 microns.

14.   The bioreactor of claim 12 wherein the entrance means is comprised of at least one macropore in the microsphere wall, each said macropore having a diameter in the range of from about 1 to 1000 microns.

15.   The bioreactor of claim 1 wherein said biocatalyst is at least one viable cellular member selected from the group consisting of animal cells, plant cells, cell masses, cell tissues, yeast, bacteria, viruses, fungi, mold and algae.

16. The bioreactor of claim 15 wherein said entrance means is comprised of at least one macropore in the microsphere wall, each said macropore having a diameter in the range of from about 5 to 200 microns.

17. The bioreactor of claim 16 wherein said biocatalyst is at least one viable animal cell selected from the group consisting of normal cells, tumor cells, fused cells, genetically engineered cells, and modified cells, and mixtures thereof.

18. The bioreactor of claim 16 wherein said biocatalyst is at least one viable microbe selected from the group consisting of bacteria and yeast, including normal microbes and microbes which have been modified by genetic engineering.

19. The bioreactor of claim 1 wherein said fluid permeable immobilizing means includes a gel present at least in the pores and entrance means of the microsphere wall.

20. The bioreactor of claim 1 wherein said fluid permeable immobilizing means comprises a continuous, semicontinuous or discontinuous permselective membrane closing-off the pores and the entrance means in the wall of the microsphere, the permeability of said membrane being such that the biocatalyst within the intracapsular space of the microsphere is prevented from escaping from the microsphere, while liquids, gases and organic molecules of predetermined molecular size which is smaller than the size of the biocatalyst, can enter or leave the intracapsular space via at least one of the pores and entrance means by passage across the permselective membrane.

21. The bioreactor of claim 1 wherein said permselective membrane is formed by interfacial polymerization within the entrance means and pores.

22. A bioreactor comprising a hollow microsphere container
and a liquid suspension or solution of biocatalyst encapsulated
within said microsphere, said microsphere comprising
a spherical porous wall comprised of sintered together
inorganic particles, said wall being of substantially
uniform thickness and porosity, said wall including interconnecting
voids which extend from the outer surface of the wall
to the inner surface of the wall, said microsphere wall
encircling an intracapsular space in the form of a single
hollow central void, said intracapsular space being at
least 70% filled with the biocatalyst solution or suspension,
said microcapsule wall further comprising a plurality
of macropore openings extending through the thickness
of the wall and having a diameter larger than the major
dimension of the biocatalyst, and a semipermeable membrane
having a thickness substantially less than the thickness
of the microsphere wall, said membrane extending across
the interconnecting voids and the macropores in order
to encapsulate the biocatalyst within the intracapsular
space, but permitting diffusion of gases, liquids and
dissolved or suspended substances having a molecular
weight below a predetermined molecular weight cut-off.

23. The bioreactor of claim 22 wherein said microsphere
has an outside diameter in the range of from about 1200
to about 6000 microns, a wall thickness of from about
10 to about 200 microns, with a ratio of wall thickness
to outer diameter in the range of from about 1:10 to
1:300, and a porosity of from about 5 to 45% exclusive
of said macropore openings.

24. The bioreactor of claim 23 wherein the macropore
openings have a diameter in the range of from about 5
to about 200 microns.

25. The bioreactor of claim 22 wherein said sintered
particles are selected from the group consisting of ceramic
particles, glass particles, metal particles and metal-glass
particles.

26. The bioreactor of claim 22 wherein said microsphere
is rigid.

Claim 27. The bioreactor of claim 26 wherein said macropore
opening is at least about 2 times the major dimension
of the biocatalyst.

28. A mass of the bioreactors of claim 1.

29. The bioreactors of claim 28 wherein each of said
microspheres forming the containers for said bioreactors
have substantially the same diameter.

30. The bioreactor of claim 29 wherein each of said
microspheres have substantially the same wall thickness
and porosity..

31. The bioreactors of claim 28 wherein said microsphere
containers are rigid.

32. The bioreactors of claim 31 wherein said biocatalyst
is at least one biochemically active material selected
from the group consisting of enzymes, antibodies, hormones,
hemoglobin, interferon and lymphokines.

33. The bioreactor of claim 31 wherein said biocatalyst
is at least one viable living cellular biocatalyst selected
from the group consisting of normal, modified, fused
and genetically engineered animal cells, yeast and bacteria.

34. A biochemical process for the production, purification,
separation, identification or quantification of a substance
wherein the substance or a precursor thereof is acted
upon by a biocatalyst to effect said production, purification,
separation, identification or quantification which comprises
carrying out the biochemical process using a plurality
of the bioreactors of claim 1 by maintaining the biocatalyst
filled microspheres in contact with a fluid medium containing
at least one low molecular weight substance which is

reactive with the biocatalyst and which can diffuse through the fluid permeable immobilizing means into the intracapsular space.

35. A biochemical cell culture process for the production of a biological substance which comprises culturing encapsulated cellular biocatalyst in a liquid culture medium using a plurality of the bioreactors of claim 33 and maintaining the bioreactors in contact with a nutrient culture medium for the cellular biocatalyst.

36. The biochemical process of claim 35 wherein the semipermeable membrane has a molecular weight cut-off size of about 200000 daltons and wherein any nutrients required for the maintenance and/or growth of the cellular biocatalyst which have a molecular weight greater than 200,000 daltons are initially present within the intracapsular space.

Fig.1

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Fig.2

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Fig.3

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Fig.4

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Fig. 5

Fig. 6
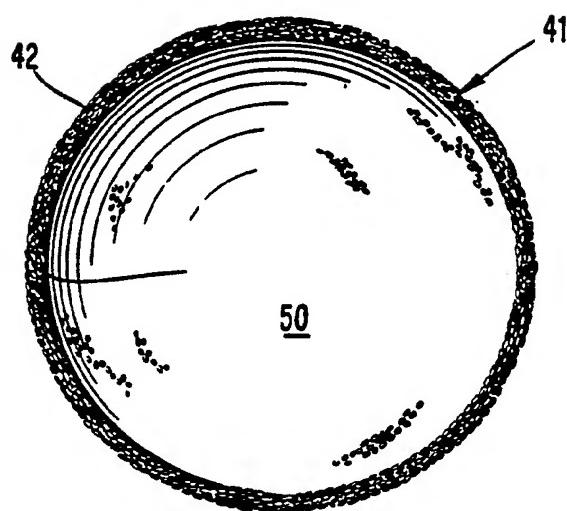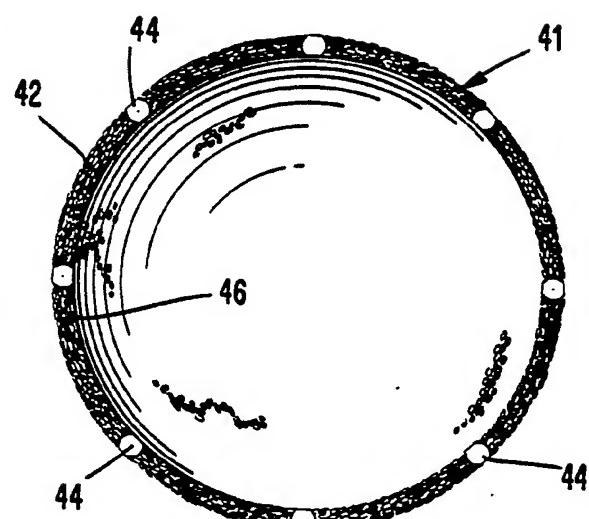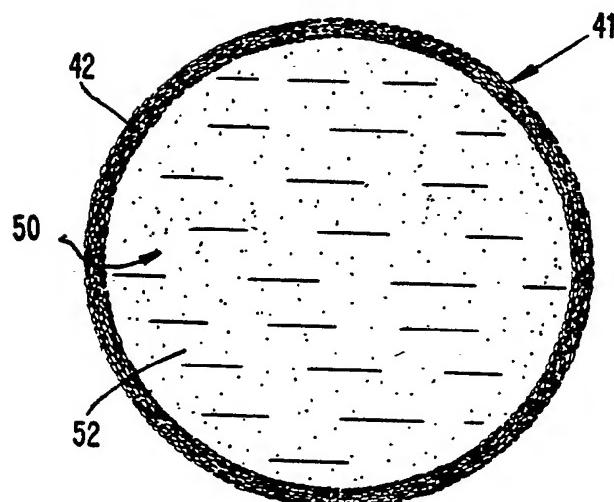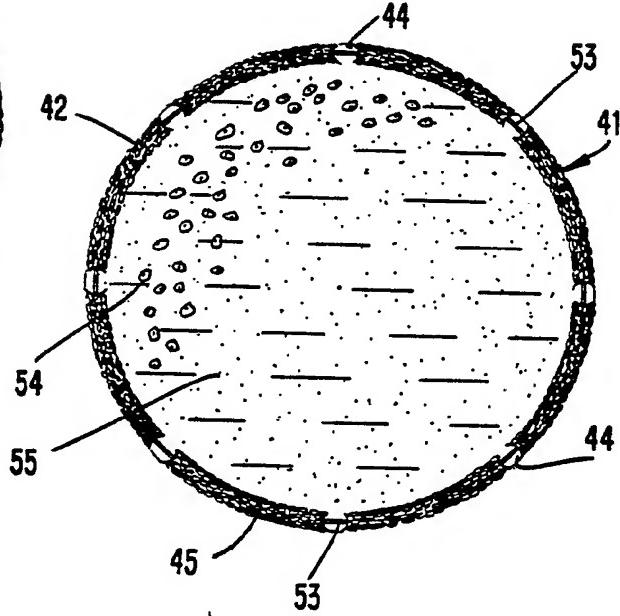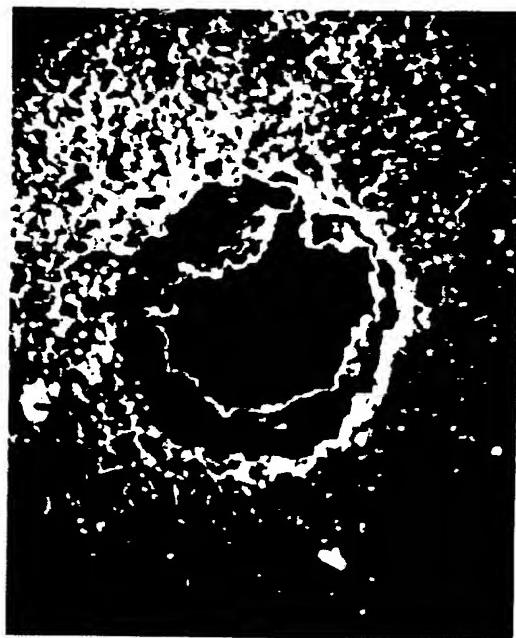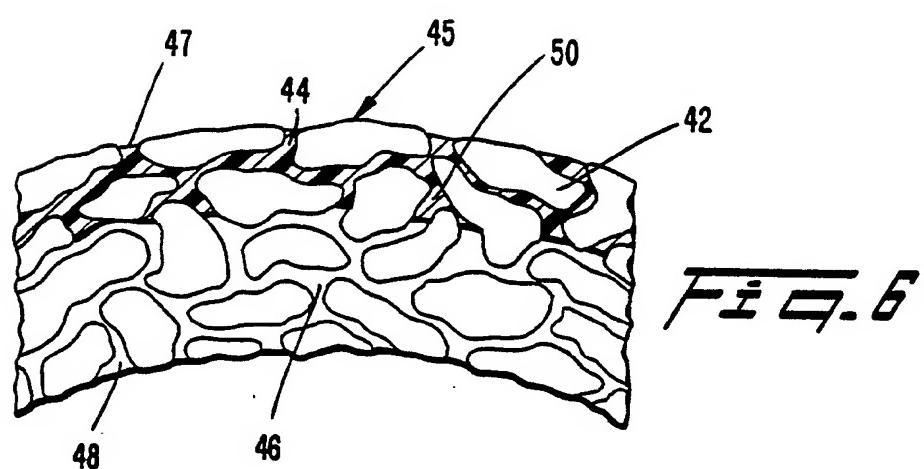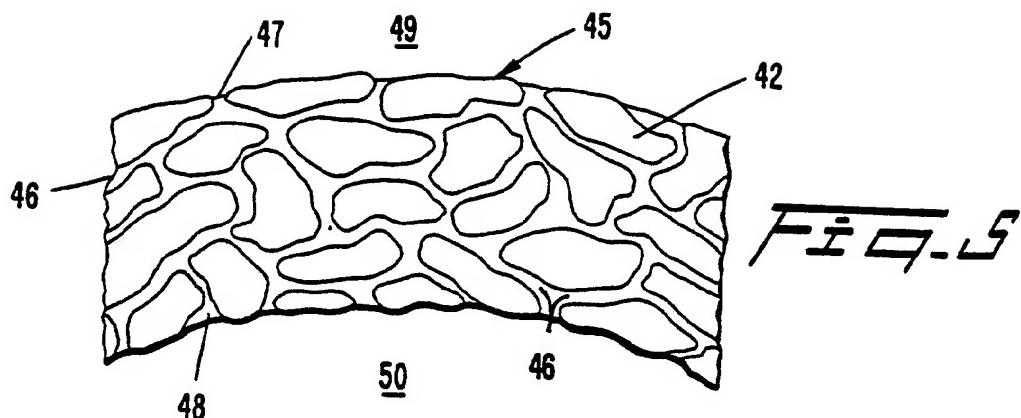
Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No　PCT/US85/01893

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. Cl.　C12P 1/00; C12N 11/14, 11/08; C12M 1/40; G01N

II. FIELDS SEARCHED, 552; B32B 5/16; B01J 37/36　see attachment

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.	435/41, 176, 178, 180, 182, 288; 436/524, 525, 527; 502/7, 8, 9, 10, 506; 428/402.2, 402.21

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 5

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
A	US,A, 4,349,456, Published 14 September 1982, Sowman	1-36
A	US,A, 4,153,510, Published 08 May 1979, Messing, et al.	1-36
A	US,A, 4,352,883, Published 05 October 1982, Lim.	1-36
A	US,A, 4,448,884, Published 15 May 1984, Henderson	1-36
A	US,A, 4,442,216, Published 04 April 1984, Harvey, et al.	1-36
A	US,A, 4,321,141, Published 23 March 1982, Messing	1-36
A	US,A, 4,275,149, Published 23 June 1981, Litman et al.	1-36

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
02 December 1985	0 4 DEC 1985
International Searching Authority 1 ISA/US	Signature of Authorized Officer 20 David M. Naff

Form PCT/ISA/210 (second sheet) (October 1981)
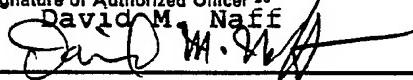
I. Classification of Subject Matter

U.S. Cl. 435/41, 176, 180, 288; 436/524, 527; 428/402.2; 502/7

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
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A	US,A, 4,303,431, Published 01 December 1981, Torobin.	1-36
A	US,A, 4,415,512, Published 15 November 1983, Torobin.	1-36
A	N, Immobilized Enzymes, John Wiley and Sons, N.Y., issued 1978, Chibata, I., pages 10, 54-40.	1-36